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(57) Abstract

A novel technique for determining the existence or nonexistence of a test nucleotide on a strand of DNA is provided. The determination advantageously uses an agent for polymerization that is capable of synthesizing an extension product if there is a match between the test nucleotide on the strand of DNA and the nucleotide opposite on an extension primer, but not if there is a mismatch. The presence or absence of the test nucleotide then may be established by determining whether the primer initiates the synthesis of an extension product in the presence of the polymerization agent. The primers of the invention may be provided with unique tails such that a single sample of DNA may be treated with a plurality of primers to test simultaneously for multiple alleles at many loci. Substrates spotted at distinct locations with oligonucleotides complementary to the unique tails of the primers may be employed to determine simultaneously the presence or absence of each of the extension products in a single test sample.

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MULTIPLEX DNA DIAGNOSTIC TEST

Background of the Invention

This invention relates to methods and products useful in detecting the presence or absence of a nucleotide at a specific location on a strand of DNA, and is therefore useful in determining an individual's genotype at any locus of interest.

A single nucleotide position on a strand of DNA may be responsible for polymorphism or allelic variation. There are known disease states that are caused by such variation at a single nucleotide position. The usefulness of detecting such variation includes but is not limited to gene typing, karyotyping, genotyping, DNA family planning, diagnostics (including infectious disease), prenatal testing, determining parentage, and forensic analysis.

The typical methods for determining such variation have included the following: (1) hybridizing specific probes to Southern blots containing different DNA digests in order to test for variation in the length of specific restriction

fragments; (2) amplifying specific regions of DNA samples by using the polymerase chain reaction (PCR) methodology, and testing for variation by sequence analysis or by hybridization with allele specific probes.

Each of these methods has certain drawbacks. For example, Southern blot analysis suffers from lack of reproducibility, the need to run gels to separate DNA fragments, and the extended amount of time required to complete the necessary steps in the process. PCR techniques suffer from occurrence of false signals arising from contamination, and the time and technical expertise required for the determination of DNA sequences from PCR amplified samples. However, perhaps the most serious drawback is that both methods require a number of separate analyses to test for variation at more than one DNA locus.

Summary of the Invention

The present invention involves a novel technique for determining the existence or nonexistence of a particular nucleotide at a particular position on a strand of DNA. The determination requires the use of a special primer capable of pairing with the strand of DNA and capable of initiating the formation of an extension

product if there is a base pair match at the nucleotide of interest. The determination advantageously employs a special agent for polymerization that is incapable of synthesizing an extension product if there is a base pair mismatch at the nucleotide of interest when the primer and DNA are paired with one another.

According to one aspect of the invention, a test sample of DNA is treated with an oligonucleotide primer of the invention, the primer being capable of pairing with a first portion of the DNA adjacent to the test nucleotide. The primer of the invention includes a nucleotide at the position opposite the test nucleotide when the primer and the DNA are paired. Then, the primer-DNA pair is treated with conditions that allow the construction of an extension product complementary to a second portion of the DNA if there is a base-pair match at the test nucleotide position, but not if there is a mismatch. Then, the presence or absence of the extension product is determined.

The method of the invention advantageously employs an agent for polymerization that is incapable of synthesizing an extension product if there is a base pair mismatch at the nucleotide of interest when the primer and DNA are paired with one another. However, other procedures may be employed

using conventional agents for polymerization. For example, an agent capable of recognizing and binding to a mismatched base pair and further capable of blocking the formation of an extension product by a conventional polymerization agent may achieve the same results. The important consideration is that conditions be applied to the paired primer-DNA strand such that an extension product will be formed in the presence of a base pair match at the test position, but not in the presence of a base pair mismatch at the test position.

The invention has the advantage of not requiring the troublesome, time-consuming gels employed in certain of the prior art detection techniques.

The extension product may be detected according to conventional methods. For example, extension nucleoside triphosphates may be labeled and the presence or absence of a labeled extension product may be assayed. Determining the presence or absence of a labeled extension product is well within the knowledge of one of ordinary skill in the art.

Preferably, the presence or absence of the extension product is determined according to a novel method that requires an oligonucleotide primer having attached to it a unique tail sequence

non-complementary with the test DNA. If an extension product is formed, that product will include the extension portion, the primer, and the non-complementary, unique tail sequence. The extension product then is applied to a substrate carrying an oligonucleotide at least in part complementary to the unique tail sequence under conditions that allow the unique tail sequence of the extension product to hybridize to the complementary oligonucleotide on the substrate. Preferably, the oligonucleotide complementary to the tail comprises repeating units of complementation to the tail. This favorably affects the kinetics of hybridization, increasing the speed and the sensitivity of the test. This novel assay may be sensitive enough to permit elimination of the multiple steps of certain of the prior art detection assays relying on DNA amplification.

The invention allows for determining genotype by testing a single sample of DNA simultaneously for multiple alleles at a single locus or for testing simultaneously for a single allele or multiple alleles at many loci. The test DNA is treated with a plurality of different oligonucleotide primers, each of the primers being complementary to a different allele and each having a unique tail. The treatment includes subjecting the primers and DNA to

conditions that allow the primers and DNA to pair. The paired primer-DNA then is treated with the polymerization agent of the invention under conditions that allow the construction of an extension product if there is a match between any test nucleotide and the opposite nucleotide on the primer, but not if there is a mismatch. The presence or absence of a particular extension product (which corresponds to the presence or absence of an allele) then may be determined by applying the sample containing the putative extension products to a substrate spotted at distinct locations with unique oligonucleotides complementary to each of the unique tails. If a particular extension product exists, then it will attach to the substrate at only one location via the hybridization of the unique tail to the unique, complementary oligonucleotide found only at that location. The presence or absence of a specific allele then may be determined by detecting the presence of an extension product at a specific location on the substrate.

The invention thus provides an oligonucleotide primer having a sequence of nucleotides complementary with at least a portion of a DNA strand and terminating at or very close to a nucleotide position opposite to a test nucleotide

position on the DNA strand where polymorphism originates due to nucleotide variation at that test position. Preferably the oligonucleotide primer has a unique tail that is non-complementary with genomic DNA. Sets of such oligonucleotide primers are provided for determining the genotype of an individual at a number of different loci.

The invention further provides a polymerization agent incapable of synthesizing an extension product in the presence of a paired primer-DNA strand having a mismatched base-pair at or close to an end of said primer. The polymerization agent of the invention may have no 5' exonuclease activity and/or may have no 3' exonuclease activity. The polymerization agent of the invention may be a native polymerase, a mutant polymerase or may be a non-mutant polymerase treated to impart the necessary properties.

The invention also provides a substrate having attached to it at one location a first oligonucleotide having a first sequence non-complementary with genomic DNA and at a second location a second oligonucleotide non-complementary with genomic DNA and also having a sequence different from and non-complementary with the first oligonucleotide. Preferably the first and second oligonucleotides have repeating nucleotide

sequences. Most preferably, the substrate is nonwettable and is spotted with oligonucleotides having repeating sequences and being firmly bound to the substrate but accessible for hybridization with complementary sequences. The substrate may have attached to it in such a manner at known locations many different oligonucleotides which are non-complementary with genomic DNA and non-complementary with each other.

The products of the invention are advantageously provided in kits. Such kits may include sets of oligonucleotide primers specific for multiple alleles at a single locus or specific for a single allele or multiple alleles at many loci. The kits also may include a plurality of different oligonucleotide primers, each of the primers having a unique tail sequence and a plurality of complementary oligonucleotides, each of the complementary oligonucleotides capable of pairing with only one of the unique tail sequences. Most preferably, the kit includes a substrate having attached to it at different locations the complementary oligonucleotides.

Brief Description of the Drawings

FIG. 1 schematically shows the synthesis of an extension product using the polymerase of the

invention when there is a match at the terminal position;

FIG. 2 schematically illustrates the absence of the formation of an extension product using the polymerase of the invention when there is a mismatch at the terminal position;

FIG. 3 schematically shows a preferred oligonucleotide primer of the invention, which primer includes a unique tail sequence;

FIG. 4 schematically shows the detection of an extension product of the oligonucleotide primer of FIG. 3;

FIG. 5 schematically shows a preferred detection substrate for detecting the labeled extension product of FIG. 3;

FIG. 6 schematically shows a set of preferred oligonucleotide primers;

FIG. 7 schematically shows a labeled extension product of the primer of FIG. 6 hybridized to a preferred substrate for determining allelic variation;

FIG. 8 shows a second set of preferred oligonucleotide primers for detecting multiple alleles at multiple loci;

FIG. 9 schematically shows a primer of the invention hybridized to phage M13mp19 (+) strand carrying a region complementary to the primer;

FIG. 10 shows the size and amount of the extension product formed by application of a polymerase* to a set of primers and test DNA; and

FIG. 11 schematically shows the preferred method for building tailed primers and detection oligonucleotides complementary to the tails, both of repeating sequences.

Detailed Description of the Drawings

The present invention involves a novel technique for determining the existence or nonexistence of a particular nucleotide on a strand of DNA, at least a portion of which strand has a known sequence. The invention may be used in connection with many medical tests, including all of those listed in the background of the invention. It is particularly useful in determining an individual's genotype, especially as the genotype relates to the existence of an allele or mutation responsible for a disease state or as it relates to an individual's identity.

The invention involves using an oligonucleotide primer that will either create a base pair match or mismatch between a test nucleotide on a DNA strand and a nucleotide on the primer opposite the test nucleotide when the primer is paired with the DNA strand. First, the primer

and DNA strand are caused to pair. Then, conditions are applied to the primer-DNA pair that will cause the formation of an extension product in the presence of a match, but not in the presence of a mismatch. The test advantageously involves the use of an agent for polymerization that is incapable of synthesizing an extension product when an extension primer and DNA are paired if there is a mismatch, but is capable of synthesizing the extension product if there is a match.

The term "oligonucleotide" as used herein in referring to primers, extension products, tails and products complementary to primers, extension products and tails, refers to a molecule comprised of two or more deoxyribonucleotides or ribonucleotides, synthetic or natural, preferably more than three. The exact size of the molecule may vary according to its particular application.

The term "primer" as used herein refers to an oligonucleotide which, when paired with a strand of DNA including a complementary sequence, is capable of initiating the synthesis of an extension product in the presence of a suitable polymerization agent. Preferably, the primer is an oligoribonucleotide and most preferably is an oligodeoxyribonucleotide. The primer, however, may be other than a ribonucleotide. The primer must be sufficiently

long to prime the synthesis of extension products in the presence of the polymerization agent. The exact length of the primer will depend on many factors, including the degree of specificity of pairing required, temperature of the annealing and the extension reactions and the source and structure of the primer.

The term "polymerization agent" as used herein may be any compound or system which will function to accomplish the synthesis of primer extension products, including enzymes. Suitable enzymes for this purpose may include DNA polymerases, reverse transcriptase, and other enzymes, including heat-stable enzymes, which will facilitate combination of the nucleotides in the proper manner to form the primer extension products which are complementary to the nucleic acid strand to which the primer hybridizes. Generally, the synthesis will be initiated at the 3' end of each primer and proceed in the 5'to 3' direction moving 3' to 5' along the template strand, until synthesis terminates. There may be agents, however, which initiate synthesis at the 5' end and proceed in the other direction.

The term "terminal nucleotide" as used herein in referring to oligonucleotide primers refers to the terminal nucleotide at the extension-initiating

end of the primer. When the primer is hybridized to the test DNA, the nucleotide position of the primer opposite to the position of the test nucleotide on the DNA strand is located at or close to the terminal nucleotide.

The term "pairing" as used herein contemplates any and all methods of sequence specific pairing between the primer and a strand of DNA including the pairing of a primer with double stranded DNA, so long as an extension product may be formed from such a pairing. Typically, however, a single stranded primer and a single strand of DNA will be paired by subjecting them to conditions which cause them to hybridize to one another. The primers are selected to be "substantially" complementary to the strands of each specific DNA sequence being tested. By substantially it is meant that the primer is sufficiently complementary to pair with the test DNA. The primer sequence then need not reflect the exact sequence of the test DNA. However, in a preferred embodiment, the primer is at least 20 nucleotides long and contains no mismatches with the test DNA strand, except in certain instances at or close to the nucleotide position opposite the test nucleotide.

The oligonucleotide primers (including tails) of the invention may be prepared using any suitable

method, such as, for example, methods using phosphotriesters and phosphodiesters well known to those skilled in the art. In one such automated embodiment, diethylphosphoramidites are used as starting materials and may be synthesized as described by Beaucage and Caruthers 1981, Tetrahedron Lett. 22: 1859-1862. One method for synthesizing oligonucleotides on a modified solid support is described in U. S. patents 4,458,066 and 4,500,707. It is also possible to use a primer which has been isolated from a biological source (such as restriction endonuclease digest of plasmid or phage DNA).

Typically, a single stranded primer when hybridized to a longer single strand of DNA in the presence of nucleoside triphosphates and an agent for polymerization at suitable temperature and pH will allow the synthesis of an oligonucleotide attached to and extending from the primer, the oligonucleotide being complementary with the single strand of DNA. Many known agents of polymerization will not incorporate a mismatched terminal base pair into an extension product. Rather, the agent of polymerization will excise the mismatched base pair by an exonuclease activity and then initiate the synthesis of an extension product from a matched pair.

According to one embodiment of the invention, the agent for polymerization does not have such exonuclease activity and is also incapable of extending from a terminal mismatched nucleotide. (Such agents herein are referred to as polymerase*). Therefore, as shown in FIG. 1, if there is complementary base pairing (a match) between the terminal nucleotide 12 of the primer 14 and the test nucleotide 16 on the test DNA 18, an extension product will be synthesized. However, as shown in FIG. 2, if there is a mismatch between the terminal nucleotide 12 of the primer 14 and the test nucleotide 16, then an extension product will not be synthesized because the polymerase* of the invention does not have the ability to initiate synthesis from a mismatch nor does it have the ability to excise the mismatch.

The synthesis of the extension product may be according to methods well-known to those skilled in the art. For example, if a deoxyribonucleotide extension product is being synthesized, the hybridized primer-DNA strand may be treated with polymerase* in the presence of deoxyribonucleoside triphosphates (dATP, dCTP, dGTP, dTTP). According to a preferred embodiment, the deoxyribonucleoside triphosphates are labeled so that if an extension product is synthesized, the existence of the

extension product may be detected easily. Typical labels include ^{32}P-labeled or biotin labeled nucleoside triphosphates.

By using a polymerase* which is incapable of synthesizing an extension product if there is a mismatch, and by subjecting the hybridized primer-DNA to conditions that would allow a labeled extension product to be synthesized if there was a match, the presence or absence of a nucleotide on a strand of DNA may be determined. For example, assume that a gene for a health trait includes the following known sequence: 3'GATCGAATTGGCACACGTT5'. Also assume the gene for the disease state is due to or correlated with a substitution at a single test nucleotide position, underlined: 3'GATCGAATTGGCCCACGTT5'. A primer that could be used to detect the presence or absence of the disease state then would be: 5'CTAGCTTAACCGG3'. This primer will hybridize with either of the foregoing DNA sequences. However, when the primer hybridizes to the DNA sequence characteristic of the healthy state, there will be a mismatch at the terminal end of the primer, an A being paired with a G. If that hybridized primer-DNA strand is treated with a polymerase* which cannot initiate the synthesis of an extension product beginning from a mismatch, then no extension product will be formed.

On the other hand, when the primer is hybridized with the DNA sequence characteristic of the disease state, there is a match between the terminal nucleotide of the primer and the test nucleotide on the DNA strand (G–C). That hybridized primer-DNA strand will initiate the synthesis of an extension product in the presence of the polymerase* of the invention because there is a match, not a mismatch.

To determine whether a sample of test DNA carries the DNA characteristic of the healthy state or the disease state, the primer is added to a sample of test DNA under conditions allowing the primer to hybridize to the test DNA. Polymerase* and nucleoside triphosphates then are added and the mixture is subjected to conditions that allow the synthesis of an extension product if there is a match between the terminal nucleotide of the primer and the test nucleotide of the DNA. It then is determined whether any DNA extension product was formed. If there is extension product, then there was a match indicating the presence of the DNA characteristic of the disease state. If there is no extension product, then there was not a match and the nonexistence of the DNA characteristic of the disease state is established.

Detection methods well known to those skilled in the art may be employed to determine the presence

of the extension product. For example, labeled nucleoside triphosphates may be used in constructing any extension product so that the extension product formed is labeled. Then, DNA complementary to the labeled extension product may be attached to a substrate such as filter paper. This substrate may be treated with the test DNA under conditions that would allow any labeled extension product to hybridize to the complementary DNA on the substrate. The substrate then would be tested for the presence or absence of any labeled extension product attached via hybridization to the complementary strand. If there is label on the substrate, then an extension product was synthesized. If there is no label on the substrate, then an extension product was not synthesized. (Alternatively, if the nucleoside triphosphates of the extension reaction are labeled with a fluorescent chromophore, then the presence of an extension product may be detected in solution by, for example, adding a fluorescent intercalating agent whose emission wavelength overlaps with the absorption wavelength of the labeled nucleoside triphosphate and measuring the nonradiative fluorescence resonance energy transfer according to the method of Cardullo et al (1988) Proc. Nat. Acad. Sci, USA 85: 8790-8794.)

The complementary DNA attached to the substrate may be complementary to at least a portion of the primer, at least a portion of the synthesized extension product, or to at least a portion of both. If the complementary DNA on the substrate is complementary to a portion of the primer, then it will be recognized by those of ordinary skill in the art that it would be preferable to separate any excess unhybridized primer from the test sample prior to applying the test sample to the substrate to prevent free primer from blocking any hybridization sites on the substrate. This may be accomplished according to conventional techniques including, but by no means limited to, separating all double stranded DNA from the free primer on a hydroxyapatite column or incorporating biotinylated nucleotides into the extension product and collecting any biotinylated DNA.

It should be understood that prior to applying labeled extension product to a substrate carrying the complementary oligonucleotide, the extension product could be amplified by, for example, PCR. PCR is described in U. S. patent 4,683,195, the disclosure of which is incorporated herein by reference. Alternatively, a linear amplification may be employed by repeating the extension reaction several times to permit more primers with matches at

the test nucleotide position to be extended with labeled nucleoside triphosphates. This may be accomplished conveniently by heat-denaturing the reaction, cooling to reanneal new primers, adding more polymerase and incubating for extension. However, if the label emits sufficient signal and if the primer is sufficiently long and sufficiently complementary with the test DNA such that the primer will hybridize only at the test location, then amplification should not be required. Preferably the primer is 20 nucleotides long and most preferably is 30 nucleotides or 40 nucleotides long. This length will ensure hybridization at the test location and extension products as short as about 100 nucleotides long may be detected.

According to a preferred embodiment of the invention, the speed and sensitivity of the test is improved by using a primer 24 having primer portion 25 and a tail portion 26 attached to and extending from the end of the primer portion 25 opposite the terminal nucleotide 12 (FIG. 3). Preferably, the tail portion 26 is unique and is non-complementary with the test DNA. Such a primer is shown schematically in FIG. 3 hybridized to a longer strand of DNA 18.

When using the primer 24 of the invention, an extension product 27, having three portions is

formed (FIG. 4). The extension product will include the extension portion 28, the primer portion 25 and the tail portion 26. If labeled nucleoside triphosphates are used, the extension portion will of course be labeled.

Improvements to the speed and sensitivity of the assay for the extension product are achieved using such primers having tails. The extension products 27 may be detected by using substrates such as filter paper, nylon, or nitrocellulose 30 spotted with a great excess of oligonucleotide complementary to the tail portion 26. Because such complementary oligonucleotide DNA 32 may be synthesized inexpensively in great quantity and therefore may be applied to the substrate in great excess (FIG. 4), the rate and amount of hybridization between the tail portion 26 of the extension product 27 and the complementary oligonucleotide 32 on the substrate is increased as compared to a system employing DNA complementary to the extension product, which DNA generally is natural DNA, is not generally available in great quantity and is usually mixed with other DNA, thereby diluting it.

Most preferably, the oligonucleotide complementary to the tail portion 26 is one having repeating units of complementation to the tail portion. Most preferably, the tail portion 26 is an

oligonucleotide 14 nucleotides long, and the complementary oligonucleotide 32 is a polymer consisting of repeating units of an oligonucleotide that is 14 nucleotides long. The use of such repeating units of complementation favorably affects the kinetics of hybridization, further increasing the speed and the sensitivity of the assay. Either the tail portion (26), or the complementary oligonucleotide (32), or both may consist of repeating units.

A substrate having attached to it a plurality of polymers 33 of such repeating units 34 of complementation is shown schematically in FIG. 5. Preferably, the substrate is nonwettable and the plurality of polymers 33 are covalently linked to the substrate at a very high concentration to form a solid solution that presents a great many available hybridization sites, unobstructed by the substrate (30) to which the polymers are attached. These substrates with attached polymers may be dried out and stored for considerable periods.

The products and methods of the invention may be used advantageously to determine allelic variation in genotyping studies. For example, if allelic variation is due to a single nucleotide substitution (or is correlated with such a substitution), then test DNA can be treated using

primers to both alleles to determine whether an individual is homozygous or heterozygous with respect to those alleles. Such a test is performed advantageously using primers having tails differing from one another so that only a single test carried out in a single vessel is necessary.

To accomplish this, two primers are constructed as shown in FIG. 6. Each primer has a primer portion P that is complementary to the same DNA, except that the terminal nucleotide on each of the primers is different. The terminal nucleotide on one of the primers is complementary to the nucleotide determining one allele and the terminal nucleotide on the other primer is complementary to the nucleotide determining a second allele. In the example shown, the terminal nucleotides are cytosine and guanosine (C and G respectively).

At the opposite end of each of the primers is attached a unique tail. By "unique" it is meant that a sequence complementary to one tail will not hybridize with the other tail. Moreover, neither of the tails and neither sequence complementary to the tails should be capable of hybridizing with the test DNA. It is believed that a single nucleotide substitution on an oligonucleotide 14 nucleotides long is sufficient to prevent cross hybridization. Preferably there are at least two nucleotide

substitutions to distinguish each tail. As is understood by those skilled in the art, the synthesis of a set of thousands of such unique tails 14 nucleotides long is possible.

The designations for the primers shown in FIG. 6 are T_1P_1C and T_2P_1G: the T signifying tail and the subdesignation signifying the sequence of the tail; the P signifying primer portion and the subdesignation signifying the sequence of the primer; and the last letter signifying the terminal nucleotide. Thus, T_1P_1C stands for tail sequence number 1, primer sequence number 1, and a cytosine terminal nucleotide. T_3P_2A would stand for tail sequence number 3, primer sequence number 2 and adenosine as a terminal nucleotide.

The primers shown in FIG. 6 (T_1P_1C and T_2P_1G) are added to test DNA under conditions that allow the primers to hybridize with the test DNA. Then the hybridized primer-DNA may be treated with polymerase* and labeled nucleoside triphosphates under conditions that allow the synthesis of a labeled extension product if there is a match at the terminal nucleotide. Thus, if the test DNA has a G at the test nucleotide, which is opposite to the terminal nucleotide of the primer T_1P_1C, then there is a match and a labeled extension product will be synthesized. Likewise, if

the test DNA has a C at the test nucleotide which is opposite to the terminal nucleotide of the primer T_2P_1G, then there is a match and a labeled extension product will be synthesized. The sample potentially containing the extension products then is applied to a substrate having spotted at different locations an oligonucleotide complementary to tail number 1 (T_1') and an oligonucleotide complementary to tail number 2 (T_2') (FIG. 7). Assuming both labeled extension products were synthesized, labeled extension product will hybridize at T_1' via hybridization of tail number 1 to the T_1' oligonucleotide and labeled extension product also will hybridize to spot T_2' via hybridization of tail number 2 to the oligonucleotide at T_2'. This would indicate a heterozygous individual. If on the other hand labeled extension product is detected only at spot T_1' then the individual carries only a G at the test nucleotide. Likewise, if labeled extension product is only detected at spot T_2', then the individual carries only a C at the test nucleotide position. Thus, the genotype of an individual at a single locus may be determined in a single test, two alleles being tested for simultaneously.

It will be understood by those skilled in the art that the genotype could have been tested by

using primers having the same tail, rather than unique tails. To accomplish this, the primers must be run separately with separate samples of test DNA. It, however, is an advantage of the invention that by using unique tails, any number of alleles or loci may be tested for simultaneously. Thus, tests for different genes and tests for multiple alleles on different genes may be accomplished simultaneously according to the invention. For example, a plurality of primers may be constructed, including primers complementary to different genes. Referring to FIG. 8, a set of primers for three genes, each gene having two alleles is shown. T_1P_1C and T_2P_1G are complementary to the same gene, but to different alleles; T_3P_2A and T_4P_2G are complementary to the same second gene, but different alleles; and T_5P_3C and T_6P_3G are complementary to a third gene, but different alleles of that gene. Each of the primers has a unique tail (T_1, T_2, T_3, T_4, T_5, and T_6). When this set of primers is mixed with a single sample of test DNA, only those primers that have hybridized to the test DNA and have matching nucleotides at the terminal end of the primer will initiate the synthesis of a labeled extension product in the presence of polymerase* and labeled nucleoside triphosphates. After the conditions for

synthesizing the extension product have been applied, the test DNA then may be contacted with a substrate having oligonucleotides complementary with the unique tails spotted at different locations on the substrate. Then, the existence of an extension product is determined by looking for the presence of label on the substrate, potentially present due to labeled extension product hybridizing via its tail to the substrate. The existence of label at a particular location on the substrate indicates that an extension product was synthesized from a primer identified by its unique tail complementary only with the oligonucleotide at one particular location. Thus, the presence or absence of each of the various genes and multiple alleles may be tested simultaneously using a single sample of test DNA.

As discussed above in connection with a conventional assay, it will readily be understood by those skilled in the art that free primer, unhybridized to any DNA, may be separated from primer that is hybridized to DNA prior to contacting the substrate to prevent the tails of the free primer from blocking the hybridization sites on the substrate. Alternatively, the complement to the tails may be spotted on the substrate in sufficient excess (preferably 5 to 10-fold) over the amount of tailed primers used that all tailed primers will be

bound. Of course, only those with a labeled extension product will produce a signal in the assay.

In selecting the primer, it is important that the presence or absence of a match at the test nucleotide determine whether an extension product is formed. It is not necessary that the primer terminate at the position opposite to the test nucleotide. The primer also may include and terminate at a position close to the position opposite to the test nucleotide. For example, the position on the primer opposite to the test nucleotide may be the penultimate position. In this instance, a match at the penultimate position still must be capable of initiating the formation of an extension product, and a mismatch at the penultimate position must be sufficient to prevent the initiation of an extension product. Preferably, the primer terminates at or within four nucleotides of the position opposite to the test nucleotide.

The foregoing description of the preferred embodiments involved the use of a polymerase* agent that is incapable of synthesizing an extension product in the presence of the base-pair mismatch at the terminal nucleotide of the primer, but is capable of synthesizing an extension product in the presence of a match at the terminal nucleotide of the primer. The polymerase* agent of the invention

preferably has been altered permanently to have no 3' exonuclease activity (or naturally lacks such activity). However, the invention contemplates other polymerization agents including those having no 5' exonuclease activity and those having no 3' exonuclease activity. The invention also contemplates polymerization agents that have substantially impaired ability to form an extension product in the presence of a mismatch, although not completely incapable of doing so. Thus, a mismatch may be detected by comparing the amount of extension product formed to the amount formed using a control having a match.

The invention also contemplates incorporation of agents which prevent removal of 3' mismatched nucleotides by a pyrophosphorolysis reaction (Rozovskaya et al., 1989, FEBS Lett. 247: 289). This reaction, which is simply the reverse of the polymerization reaction, may occur at high pyrophosphate concentrations, whether or not a 3' exonuclease is present. The addition of a purified, nuclease-free pyrophosphatase to the polymerase reaction will reduce the pyrophosphate concentration to a level at which it cannot drive the pyrophosphorolysis back-reaction to remove mismatched 3' nucleotides. Thus, the sensitivity of the test will be improved.

It is not intended that the invention be limited to a polymerization agent having a particular modification, but rather it is intended that the invention include any polymerization agent having the capacity to synthesize an extension product in the presence of a paired primer-DNA strand having a match at or close to an end of the primer, but not if there is a base-pair mismatch. Such a polymerization agent may be of a mutant variety or may be a substantially pure preparation of an existing polymerization agent having the desired qualities (such as AMV reverse transcriptase).

It further should be understood that the method of the invention does not require a special polymerization agent. It is important only that a primer be selected and paired with a DNA strand, and that conditions be applied such that an extension product is formed if there is a match at the test nucleotide, and not formed if there is a base-pair mismatch at the test nucleotide. For example, the paired primer-DNA strand first may be treated with a molecule that recognizes and binds to a base-pair mismatch, that molecule being capable of blocking the initiation of the synthesis of an extension product by an otherwise normal polymerization agent. Likewise, temperature, pH, ionic strength

and the like may be varied in a manner that prevents an otherwise normal polymerization agent from initiating the synthesis of an extension product in the presence of a mismatch or further improves the discrimination of a polymerase* agent between a match and a mismatch. Thus, rather than simply providing a particular primer or a particular polymerization agent, the invention provides a system for determining the presence or absence of a test nucleotide at any particular position on a strand of DNA.

The invention may be employed to detect allelic variation or polymorphism due to a single base substitution on a strand of DNA. Such nucleotide variation is known to be responsible for particular disease states, including beta thalasemia, hemophilia, sickle cell anemia, and Familial type III hypercholesterolemia. Such nucleotide variation also is known to be responsible for polymorphism, including polymorphism known to exist as restriction length fragment polymorphism (RFLP). Lench, Stanier and Williamson 1988, The Lancet, June 18, pp. 1356-8.

The invention may be employed advantageously to determine parentage or to establish a genetic "fingerprint" that identifies one and only one individual. To accomplish this, restriction

fragment length polymorphisms (RFLPs) first are identified. Preferably, two polymorphisms, each present at signifcant frequencies in the population at large, are identified for each chromosome, the polymorphisms being located at opposite ends of each chromosome. Then, primers according to the invention are synthesized to recognize the nucleotide variation responsible for the polymorphism. Each primer is provided with a unique tail. A substrate is spotted at distinct locations with oligonucleotides complementary to each of the unique tails. Next, the labeled extension product is separated from the free primer and the labeled extension product then is applied to the substrate under conditions that would allow the labeled extension product to hybridize to the substrate via the unique tails. Then, the set of primers are paired with a test sample of DNA, and conditions are applied that would allow the formation of labeled extension products if there is a match at the test nucleotide, but not if there is a mismatch. Finally, the presence or absence of labeled extension product on the substrate is determined for each location, the existence or nonexistence of labeled extension product at these locations indicating the individual's genotype. It is believed that this genotype will be unique for each

individual if the existence of a polymorphism at each end of each chromosome is tested for. However, it will be recognized by those of ordinary skill in the art that a lesser number of loci may be tested for in determining, for example, parentage when a sample of the DNA of the mother, father and child is available.

While the foregoing examples have been described in connection with polymorphism due to single nucleotide variation, it should be readily understood that the principles apply to deletions as well.

An alternative method of detecting the existence of an extension product or to enhance the method described above involves back-strand tagging. First, according to the invention, an extension product is formed if there is a match at the test nucleotide. Then, a primer complementary to the extension product is paired with the extension product and conditions are applied to allow the synthesis of a second labeled extension product from the primer to the first extension product. The presence of this second labeled extension product may alone be determined, or may be determined in addition to the first extension product.

Example 1

Assay of purified polymerases for the ability to carry out the extension reaction of the invention.

The following assay represents one way to measure the suitability of a polymerase for practice of this invention. The assay detects the presence or absence of nucleotide addition to an oligonucleotide primer hybridized to a template strand. A polymerase suitable for practice of this invention (ie., polymerase*) will fail to incorporate nucleotides when the oligonucleotide contains a mismatch at the terminal 3' position, or both of the terminal 3' dinucleotide positions, but will incorporate nucleotides when the primer contains no mismatch or only one mismatch at the 3' penultimate position relative to the hybridizing sequence. The key feature of a polymerase* is its ability to discriminate between a single mismatch vs. a perfect match between the primer and the template, or between two consecutive mismatches vs. a single mismatch at either the ultimate or penultimate 3' position, and to extend the primer only if the latter situation of each pair obtains.

Four different 17 base pair primers were used to test nucleotide incorporation or lack of incorporation using a template consisting of phage

M13mp19 viral (+) strand carrying a "polylinker" insert (FIG. 9) (Yanisch-Perron et al, 1985, Gene 33:103-119). The primers were obtained from Operon Technologies (San Pablo, CA) and had the following DNA sequence:

Primer 1: 5' ATTCGAGCTCGGTACCC 3'

Primer 2: 5' ATTCGAGCTCGGTACCG 3'

Primer 3: 5' ATTCGAGCTCGGTACGG 3'

Primer 4: 5' ATTCGAGCTCGGTACGC 3'

Primer 1 is perfectly complementary to the template and terminates in the middle of a MspI restriction site (5'CCGG3'). Primer 2 differs from primer 1 at the 3' terminal nucleotide to produce a G:G terminal mismatch upon hybridization to the template. Primer 3 differs from primer 1 at the 3' pair of nucleotides to produce a GG:GG double mismatch upon hybridization to the template. Primer 4 differs from primer 1 at only the penultimate 3' residue to produce a G:G penultimate mismatch upon hybridization to the template. Since many randomly isolated RFLP probes detect polymorphisms at MspI restriction sites (Donis-Keller et al., 1987, Cell 51: 319-337), these primer-template sets mimic a typical situation which occurs naturally at many human loci and which could be determined by the test

of this invention.

The 5' ends of the primers were labeled with ^{32}P according to the method of Maniatis et al. (1982, Molecular Cloning — A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, pp.125). About 20 pmoles of each primer were labeled by incubation with T4 polynucleotide kinase (NEB, Beverley, MA) and 70 uCi of [gamma-^{32}P]-ATP (3000 Ci/mmole) (NEN, Boston, MA) in a 20 ul reaction volume. The labeled primers were separated from the remaining ATP by gel filtration chromatography over a beaded polyacrylamide column Biogel P2 BioRad Laboratories, Richmond, CA) after the addition of 1 ul of "stop mix" consisting of 10 mg/ml of bovine serum albumin (previously stained with Coomassie brilliant blue) in 100 mM EDTA (pH 7.5). The primers which flowed through in the void volume were precipitated with 3 volumes of ethanol and 1/10 volume of 3M NaOAc. The precipitated labeled primers were redissolved in 20 ul of TE buffer (pH 7.9).

The assay mixture for each primer contained 0.2ug (about 0.1 pmole) of M13mp19 viral single-stranded DNA, 50 nM labeled primer, 50 mM HEPES buffer at pH 7.8, 20 mM KCl, 10 mM $MgCl_2$, 2 mM DTT, 100 ug/ml bovine serum albumin, 10 uM dATP, 10 uM dGTP, 10 uM dTTP, and 10 uM dCTP in a total

volume of 20 ul. The end-labeled primer and template were heated for 2 min at 65°C, hybridized at 37°C for 15 min, and then cooled to 16°C for 2 min. Finally, 1 ul (1 unit) of the polymerase, either Sequenase version 2.0 or T7 polymerase (US Biochemicals products #70775 & #70017, respectively, Cleveland, Ohio), was added, and the reaction was allowed to proceed for 10 min at 16°C.

Each reaction was stopped by addition of an equal volume of buffer-equilibrated phenol and 30 ul of 0.6 M NaOAc. The DNA was then extracted with 50 ul of 24:1 $CHCl_3$:isoamyl alcohol and was precipitated by addition of ethanol to 70% and incubation at -20°C. The precipitated DNA was collected by centrifugation and redissolved in 20 ul of the buffer recommended by the manufacturer for digestion with restriction endonuclease HindIII (NEB, Beverley, MA). Following digestion with HindIII, in order to produce an extension product of defined length (17 nt primer + 33 nt extension to the HindIII site = 50 nt, see FIG. 9), the reaction was extracted with phenol, and the DNA was ethanol precipitated as described above. The DNA was collected by centrifugation, redissolved in gel buffer (95% formamide, 10 mM EDTA (pH 7.5), 0.01% bromphenol blue, 0.01% xylene cyanol) and subjected to electrophoresis in an 8% polyacrylamide

sequencing gel prepared as described by Maniatis (1982, supra, p. 478). DNA size standards were also run on the gel. These were prepared by labeling MspI-cut pUC19 DNA with [alpha-^{32}P]-dCTP (3000 Ci/mmole) (NEN, Boston, MA) and Sequenase version 2.0 (US Biochemicals, supra). Sizes of the standards are 501 + 489, 404, 331, 242, 190, 147, 110 + 111, 67, 34, and 26 nucleotides. After electrophoresis, the gel was covered with Saran wrap and exposed to x-ray film for 2 hrs. FIG. 10 is a photograph of the developed x-ray film.

The size and amount of extension products synthesized by the polymerases acting on the primers and template of this example are shown in FIG. 10 by the position in the gel and the intensities of the bands in lanes 1-5. The DNA size markers are shown in lane 6. The extension products resulting form the action of Sequenase, version 2.0 on primers 1-4 are shown in lanes 1-4, respectively. The intensities of the bands in lanes 1-3 clearly indicate that much less extension product was made from primer 2 (which has an ultimate 3' position mismatch) and from primer 3 (which has both ultimate and penultimate 3' position mismatches) than from primer 1, which contains no mismatches with the template. The small amount of extension product made in lanes 2 and 3 appears to result from

slippage of the primer to allow the mismatched guanine of the primer to match with the cytosines at the next two positions of the template to produce extension products that are one or two nucleotides shorter than the expected product. This can be prevented by designing the primers in such a way that the mismatches do not match the next nucleotide following the primer hybridization site. Primer 4, having a single mismatch at the penultimate position, also yields significantly less extension product than seen from primer 1, but yields more extension product than seen from primer 3 which has two mismatches. In lane 5 is shown the extension product of T7 polymerase, which contains a 3' exonuclease activity, in the presence of primer 3 containing two mismatches. Extension appears to be efficient producing a 50 nt product resulting from trimming away the mismatched nucleotides and adding 35 nt onto the trimmed 15 nt perfectly matched primer.

Thus, Sequenase version 2.0 appears to be a suitable polymerase* for the practice of this invention, but T7 polymerase is not suitable because of its nuclease activity which removes mismatched nucleotides. According to this invention, the presence of a guanine at the test position of the template could be discerned by the use of Sequenase,

version 2.0 (polymerase*) and either primers 1 and 2, or primers 3 and 4. Analysis of the extension products resulting from the use of either pair of primers reveals that the template contained a guanine at the test position.

Other known polymerases lacking 3'exconuclease that may work well for mismatch extension discrimination include: AMV reverse transcriptase (Skinner and Eperon, 1986, Nucl. Acids Res. 14: 6945-6964), modified T7 DNA polymerase (Tabor and Richardson, 1987, Proc. Nat'l. Acad. Sci. USA 84: 4767-4771), the C-terminal domain of DNA polymerase I Klenow fragment (Freemont et al., 1986, Proteins 1: 66-73), and DNA polymerase beta (Abbotts et al., 1988, in "DNA Replication and Mutagenesis" (R. Moses & W.C. Summers, eds) Am. Soc. for Microbiol., Washington, DC). Adjustment of the concentration of assay components such as dNTPs, divalent cations, buffer components, and salts may be needed for maximum discrimination of matched and mismatched 3'-OH termini.

Example 2

Method for altering a polymerase or polymerase* to improve its discrimination between matched and mismatched 3'OH termini of primers

A cloned segment of the E.coli DNA polymerase I gene encoding the C-terminal portion of the enzyme is sufficient to produce a variant of DNA polymerase I containing polymerase activity but lacking all exonucleolytic activity (Freemont et al., 1986, supra). This fragment encoding Asp515-His928 (see sequence of Joyce et al., 1982, J. Biol. Chem. 257: 1958-1964)is removed from plasmid pCJ122 (see Derbyshire et al., 1988, Science 240: 199-201) by restriction with XhoII and cloned into a BamHI site introduced into the regulated lambda P_L expression vector pAS1 (see Rosenberg & Shatzman, 1983, Methods in Enzymology 101: 833) by use of oligonucleotide linkers. The resulting plasmid is mutagenized by a method such as the random gap misrepair method of Shortle & Lin (1985, Genetics 110: 539-555) and Shortle et al. (1982, Proc. Nat'l. Acad. Sci. USA 79: 1588-1592). The mixture of resulting modified plasmids is amplified by transforming E. coli HB101, growing the transformants, and isolating the plasmid DNA. The segment encoding the C-terminal fragment of polymerase I is removed by restricting with XhoII, and it is inserted into the NcoI site of plasmid pCGS681 by use of oligonucleotide adapters as described by Moir & Dumais (1987, Gene 56: 209-217) so that the invertase secretion signal is in translational reading frame with codons for Met

and Glu, followed by the DNA polymerase I codons introduced. Note that both orientations of the DNA polymerase I gene fragment will occur. The desired orientation, with the codon for Asp515 adjacent to the Met and Glu codons following the invertase secretion signal, is found by restricting plasmid DNA from several transformants with HindIII and SacI and identifying an approximately 200 bp fragment on ethidium bromide-stained agarose gels.

The resulting plasmid includes a strong yeast promoter from the triosephosphate isomerase gene, the yeast invertase secretion signal, and features for autonomous growth in E. coli and yeast. When used to transform yeast strain CGY1585 (MAT alpha ura3-52 leu2-3,112 ssc1-1) to uracil prototrophy, this plasmid directs the production and secretion of a fragment of E. coli DNA polymerase I. CGY1585 and other suitable yeast host strains carrying mutations in the SSC1 (also known as the PMR1) gene are described in Smith et al. (1985, Science 229: 1219-1224) and in Rudolph et al. (1989, Cell, in press) and in European Patent Application EP 201208.

Colonies secreting a mutant polymerase* with the desired properties are identified by the following protocol. Transformed yeast colonies are replica plated in quadruplicate onto fresh agar

plates lacking uracil. After these plates are grown up, each is replica plated to a circular nitrocellulose membrane which is slightly smaller than the petri plate. Each of these nitrocellulose membranes carrys the M13mp19 single-stranded DNA template bound to it by standard methods (Maniatis et al., 1982, supra, p. 331), and each has one of the four primers of Example 1 annealed to it prior to replica plating. The cells are then washed off with TE buffer containing 0.5 M NaCl, leaving the secreted polymerase bound to the membrane according to the procedure of Rothman et al. (1986, Proc. Nat'l. Acad. Sci. USA 83: 3248-3252).

The four membranes are then each incubated in a different reaction mixture containing buffer (as in the assay mixture of Example 1), dNTPs including about 70 uCi [alpha-^{32}P]dCTP at 3000 Ci/mmole (NEN, Boston, MA) without any unlabeled dCTP. After incubation for 30 min at 37^{o}C, the filters are rinsed in 10X SSC (Maniatis et al, 1982, supra, p.447) to remove reaction components, dried, and exposed to X-ray film to reveal colonies secreting polymerase capable of extending primer 1 but less capable of extending primer 2, and/or capable of extending primer 4, but less capable of extending primer 3. Such a colony will produce a strong signal on the film exposed to the nitrocellulose

membrane containing primer 1, but a weak signal with primer 2, or a strong signal with primer 4, but a weak signal with primer 3. The colony producing the desired polymerase* is removed from the original petri plate and grown in standard yeast SD medium (Sherman, Fink and Hicks, 1986, Methods in Yeast Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) supplemented with 0.4 M ammonium phosphate and buffered to pH 6.0 to produce large amounts of the desired polymerase* for use in the practice of this invention. Purification of the secreted polymerase* from the culture broth is accomplished by minor modification of the method of Kelley and Stump (1979, J. Biol. Chem. 254: 3206-3210).

For expression in other host cells, DNA is prepared from the yeast transformant cells according to the method of Sherman, Fink and Hicks (1986, supra, pp. 125-126). The resulting DNA is used to transform E. coli HB101 to ampicillin resistance, and a transformant colony carrying the desired plasmid is isolated. The plasmid DNA is amplified by growing the E. coli strain, harvesting and isolating the plasmid DNA. The DNA segment encoding the desired polymerase* is removed and expressed in any host organism desired, such as CHO cells, E. coli, P. pastoris, by providing the necessary

elements for maintenance of a plasmid and transcription of the DNA.

Example 3

An assay for a nucleotide substitution in the human beta globin gene causing sickle cell anemia.

The assay uses the following two synthetic 40-mer primers which are obtained by special order from Operon Technologies (San Pablo, CA):

primer 1: 5' TTCACCTTGCCCCACAGGGCAGTAACGGCAGACTTCTCCT 3'
primer 2: 5' TTCACCTTGCCCCACAGGGCAGTAACGGCAGACTTCTCCA 3'.

Primer 1 is perfectly homologous to the normal beta globin allele but has a 3' terminal T:T mismatch with the sickle cell allele, and primer 2 is prefectly homologous with the sickle cell beta globin allele but has a 3' terminal A:A mismatch with the normal beta globin allele. These primers avoid the potential for cross hybridization with the delta globin gene (which is highly homologous to beta globin) because they hybridize to the beta globin gene at a region in which there are 5 nucleotide differences between the two genes.

Separate extension reactions are carried out with each primer and with each human DNA sample using the assay mixture and conditions described in the assay reaction of Example 1 with the Sequenase version 2.0 (polymerase*), except that 5 ug of human DNA is used as the template, the primers are not

end-labeled, 40 uCi of [alpha-^{32}P]dCTP at 3000Ci/mmol without any unlabeled dCTP is used as the source of label, and the temperature for primer annealing is 65^{o}C. The human DNA is obtained from human cell line GM2340A which is homozygous for the sickle cell allele (HbS/HbS) and from normal human cell GM6167 (both obtained from the NIGMS Human Genetic Mutant Cell Repository, Camden, NJ) by the procedure of Bell et al. (1981, Proc. Nat'l. Acad. Sci. USA 78: 5759-5763).

Following the reaction, the presence or absence of an extension product is determined as follows. The samples are passed over a column of bead polyacrylamide (Biogel P60, Biorad, supra) column to remove excess nucleotides, and incubated for 5 min at room temperature after addition of 1/10 volume of 1 M NaOH. Next, they are hybridized by with the following 30-mer oligonucleotide ("detection oligo") which is itself first bound to four different nylon membranes:
5'ACCTCAAACAGACACCATGGTGCACCTGAC3'.

In order to bind this detection oligonucleotide to a nylon membrane without hindering its ability to hybridize to the extension product of the test reaction, the oligonucleotide is first treated with calf thymus terminal deoxynucleotidyl transferase and dGTP according to

the procedure of Maniatis et al. (1982, supra, pp. 239-240). The resulting "detection oligo" containing a poly-dG tail is then bound to discrete spots on two different nylon membranes (ZetaProbe, BioRad, Richmond, CA) by use of a dot-blot device and procedure of the manufacturer (BioRad, Richmond, CA) . The "detection oligo" is homologous to nucleotides in the beta globin gene that will be attached to the primer only if the primer has been extended by polymerase* in the reaction.

Nylon membrane number 1 is hybridized with the sample resulting from the reaction with primer 1 and GM2340A DNA, nylon membrane number 2 with the sample resulting from primer 1 and GM6167 DNA, nylon membrane number 3 with the sample resulting from primer 2 and GM2340A DNA, and nylon membrane number 4 with the sample resulting from primer 2 and GM6167 DNA. The hybridization reaction is carried out for 30 min at 60°C in 5 ml of 1M NaCl, 0.1M Tris-Cl, pH 7.5, 1 mM EDTA, 10ug/ml Heparin, 100 ug/ml single-stranded sonicated salmon sperm DNA, and 0.1% sarcosyl after pretreatment of the membrane under the same conditions in the absence of the sample for 30 min. The membranes are then washed in 2X SSC followed by 0.2X SSC at 50°C to remove non-hybridized components, and exposed to x-ray film.

A much stronger signal generated with primer 1 than with primer 2 indicates two normal alleles in the human DNA tested, a much stronger signal generated with primer 2 than with primer 1 indicates the presence of two sickle cell alleles, and an equally strong signal generated from both primers indicates that the DNA sample is heterozygous for the two alleles. In this example, the human DNA from the normal cell line GM6167 which is homozygous for the normal beta globin gene gives a much stronger signal with primer 1 (membrane #2) than with primer 2 (membrane #4), and the DNA from the homozygous sickle cell disease cell line GM2340A gives a much stronger signal with primer 2 (membrane #3) than with primer 1 (membrane #1)

Example 4

Assay for the presence of specific alleles differing by a single nucleotide using primers with tails

This example uses the same primers as described in Example 3 except that an oligonucleotide chain having the sequence $[5'GCGAGAGGAGAGCG3']_n$ (where n is greater than about 10) is added to the 5' end of primer 1 as a tail, and an oligonucleotide chain having the sequence $[5'GCGTGTGGTGTGCG3']_n$ (where n is defined

as above) is added to the 5' end of primer 2 as a different tail.

Tail addition to primer 1 is accomplished by using a tail oligonucleotide of sequence corresponding to the repeating unit of the tail along with two additional oligonucleotides (FIG. 11). The first additional oligonucleotide, 5'CTCTCGCCGCTCTC3', is a staggered complement to the tail oligo consisting of the complement of the first seven nucleotides of the tail oligo for primer 1 fused to the complement of the last seven nucleotides of the tail oligo for primer 1. In the presence of the tail oligo for primer 1, this first additional oligo will hybridize and hold two such tail oligos together (FIG. 11). The second additional oligonucleotide, 5'AGGTGAACGCTCTC3', is a tail-primer bridging oligonucleotide consisting of the complement of the first seven nucleotides of primer 1 fused to the complement of the last seven nucleotides of the tail oligo for primer 1. In the presence of the tail oligo for primer 1 and primer 1 itself, this tail-primer bridge oligonucleotide will hybridize and bridge the tail oligo to the primer (FIG. 11). The tail oligo and primer 1 are phosphorylated on their 5' ends with T4 polynucleotide kinase according to the procedure of Maniatis et al. (1982, supra, p. 125). The

staggered tail complement and the primer-tail bridge, however, are not 5'-phosphorylated, and therefore, they cannot be joined by DNA ligase. They are included in the reaction mix with the 5'-phosphorylated tail oligo and the 5'-phosphorylated primer such that the first is equimolar with the tail and the second is equimolar with the primer. The tail and primer are present at about a 40:1 molar ratio. The reaction mix is in ligation buffer (Maniatis et al., 1982, supra, pp. 125-126) and includes T4 DNA ligase. After four hours at 16°C, the reaction is terminated by heating to 65°C for 15 minutes, and remaining unligated oligonucleotides are removed by agarose gel filtration chromatography on Sepharose CL6B (Pharmacia, Piscataway, N.J.) in the presence of 50% formamide followed by ethanol precipitation.

A similar reaction is carried out with primer 2, its tail oligonucleotide (5'GCGTGTGGTGTGCG3'), staggered tail complement 5'CACACGCCGCACAC3', and primer-tail bridge 5'AGGTGAACGCACAC3' to produce a tailed primer 2 molecule which is isolated as described for the tailed primer 1.

Polymerized complements to the two primer tails are produced by ligating together, in separate reactions, equimolar amounts of the tail oligonucleotides and their staggered-tail

complements (FIG. 11). For example, the tail for primer 1 is incubated with its staggered-tail complement in a ligation reaction in which the tail oligonucleotide has a 5' hydroxyl and the staggered-tail complement oligonucleotide has a phosphorylated 5' end. In an analogous fashion, the poly-complement to the tail for primer 2 is built by ligating together 5'-phosphorylated tail complement units, 5'pCACACGCCGCACAC3', in the presence of the non-phosphorylated tail for primer 2. After four hours at 16°C, the reaction is terminated by heating to 65°C for 15 minutes, and remaining unligated oligonucleotides are removed by chromatography on Sepharose CL6B (Pharmacia, supra) in the presence of 50% formamide followed by ethanol precipitation.

At least 10 pmoles of each of the two poly-complements to the two tails are each bound at separate, discrete, known locations to the same nylon membrane (ZetaProbe, BioRad, Richmond, CA) by using conditions recommended by the manufacturer. Two such nylon membranes, each containing both poly-complements, are prepared -- one for testing the reaction with GM2340A DNA and one for testing the reaction with GM6167 DNA.

For each human DNA sample, a single extension reaction is carried out with both tailed primers

using the assay mixture and conditions described in the assay reaction of Example 1 with Sequenase version 2.0 (polymerase*), except that 5 ug of human DNA is used as the template, the primers are not end-labeled, about 1 pmole of each primer is added, 40 uCi of [alpha-^{32}P]dCTP at 3000Ci/mmol without any unlabeled dCTP is used as the source of label, and the temperature for primer annealing is 65^{o}C. Two such extension reactions are carried out in separate tubes, one with each of the two human DNA samples. The human DNA is obtained from human cell line GM2340A which is homozygous for the sickle cell allele (HbS/HbS) and from normal human cell GM6167 (both obtained from the NIGMS Human Genetic Mutant Cell Repository, Camden, NJ) by the procedure of Bell et al. (1981, supra).

Following the reactions, the presence or absence of extension products is determined as follows. The samples are passed over a Biogel P60 column to remove excess nucleotides, and incubated for 5 min at room temperature after addtion of 1/10 volume of 1 M NaOH. Next, they are hybridized to the nylon membranes each of which already has bound to it, at known, distinct positions, a 10-fold excess over the starting primers of polymerized 14-mer complementary to the tail of primer 1 and complementary to the tail of primer 2. The

hybridization reactions are carried out for 30 min at 60°C in 5 ml of 1M NaCl, 0.1 M Tris-HCl, pH 7.5, 1 mM EDTA, 10ug/ml Heparin, 100 ug/ml single-stranded sonicated salmon DNA, and 0.1% sarcosyl after pretreatment of the membrane under the same conditions in the absence of the sample for 30 min. Membrane #1 is hybridized with the products of the reaction with GM6167 DNA, and membrane #2 is hybridized with the products of the reaction with GM2340A DNA. The membranes are then washed in 2x SSC followed by 0.2x SSC at 50°C to remove unhybridized material and exposed to x-ray film in the presence of an intensifying screen at -80°C.

Development of the x-ray film reveals a spot on membrane #1 at the position of the poly-complement of the primer 1 tail which is much more intense than the spot at the position of the poly-complement of the primer 2 tail. This is consistent with the fact that the sample is human DNA from cell line GM6167 carrying two normal alleles of beta globin. The spot on membrane #2 at the position of the poly-complement of the primer 2 tail is much more intense than the spot at the position of the poly-complement of the primer 1 tail. This is consistent with the fact that the sample is human DNA from cell line GM2340A carrying two sickle cell disease globin alleles.

Example 5

Detection of multiple alleles of a single locus in a single reaction mix

Human DNA is tested for the presence of the wild-type allele and four mutant alleles of the beta globin gene in a single reaction mixture by using the following method. Mutations at several positions in the human beta globin gene are known to occur at various frequencies in specific populations, severely reducing the levels of beta globin and producing a condition known as beta-thalassemia (S.H. Orkin, 1987 "The Molecular Basis of Blood Diseases", Stamatoyannopoulos, Nienhuis, Leder & Majerus, eds., W.B. Sanders Co., Philadelphia, pp. 106-126.). Amselem et al, (1988, Am. J. Hum. Genet. 43: 95-100) have tested for four of these mutations by using a polymerase chain reaction, dot blot procedure. This example demonstrates use of the principles of the present invention to test for the same four beta-globin alleles.

DNA is derived from the following four different human cell lines carrying the following beta globin alleles by using the method of Bell et al. (1981, supra): cell line GM6167 is normal for the beta globin locus; cell line GM7407A is heterozygous for mutations IVS-1,1 and IVS-1,6; cell

line GM7405 is homozygous for mutation IVS-1,110; and cell line GM7425 is heterozygous for mutations IVS-1,110 and Codon 39 nonsense.

The synthetic oligonucleotides of TABLE 1 and TABLE 2 are obtained by special order from Operon Technologies (San Pablo, CA).

TABLE 1
Primers for analysis of beta-thalassemia alleles

Locus	Primer
IVS1,1	
A	5'ATTGGTCTCCTTAAACCTGTCTTGTAACCTTGATACCAAC3' match with normal; C:A mismatch with mutant
B	5'ATTGGTCTCCTTAAACCTGTCTTGTAACCTTGATACCAAT3' match with mutant; T:G mismatch with normal
IVS1,6	
C	5'TTTCTATTGGTCTCCTTAAACCTGTCTTGTAACCTTGATA3' match with normal; A:C mismatch with mutant
D	5'TTTCTATTGGTCTCCTTAAACCTGTCTTGTAACCTTGATG3' match with mutant; G:T mismatch with normal
IVS1,110	
E	5'AGGGTAGACCACCAGCAGCCTAAGGGTGGGAAAATAGACC3' match with normal; C:A mismatch with mutant
F	5'AGGGTAGACCACCAGCAGCCTAAGGGTGGGAAAATAGACT3' match with mutant; T:G mismatch with normal
Codon 39	
G	5'CAGGAGTGGACAGATCCCCAAAGGACTCAAAGAACCTCTG3' match with normal; G:T mismatch with mutant
H	5'CAGGAGTGGACAGATCCCCAAAGGACTCAAAGAACCTCTA3' match with mutant; A:C mismatch with normal

TABLE 2

Oligonucleotides to be polymerized to become the tails and the complement to the tails

Tail Unit:		Staggered Tail Complement Unit:		Tail-Primer Bridge:	
A'	5'GCGCACGGCACGCG3'	A"	5'CGTGCGCCGCGTGC3'	A#	5'GACCAATCGCGTGC3'
B'	GCGCTCCCCTCGCG	B"	CGAGCGCCGCGAGG	B#	GACCAATCGCGCGG
C'	CGCACACCACACGC	C"	GTGTGCGGCGTGTG	C#	ATAGAAAGCGTGTG
D'	CGCTCTCCTCTCGC	D"	GAGAGCGGCGAGAG	D#	ATAGAAAGCGAGAG
E'	GCAACGGGGCAACG	E"	CCGTTGCCGTTGCC	E#	CTACCCTCGTTGCC
F'	GCTTCCCCCCTTCG	F"	GGGAAGCCGAAGGG	F#	CTACCCTCGAAGGG
G'	GCATCCCCCCTAGC	G"	GGGATGCCGTAGGG	G#	ACTCCTGCGTAGGG
H'	CGTCCCAACCCTGC	H"	TGGGACGGCAGGGT	H#	ACTCCTGGCAGGGT

Tails are added to primers as follows. The 5'-phosphorylated tails (A'-H', TABLE 2) for each primer (A-H, TABLE 1) are polymerized onto the 5' end of the appropriate 5'-phosphorylated primer as described in Example 4 (FIG. 11) above by using T4 DNA ligase and the appropriate non-phosphorylated staggered-tail complement unit (A"-H", TABLE 2) and non-phosphorylated tail-primer bridge oligonucleotides (A#-H#, TABLE 2). After four hours at 16°C, the reaction is terminated by heating to 65°C for 15 min, and remaining unligated oligonucleotides are removed by chromatography on Sepharose CL6B (Pharmacia, supra) in the presence of 50% formamide followed by ethanol precipitation.

Polymerized complements to the tails are constructed as follows. Oligonucleotides (A"-H", TABLE Y) which are the staggered tail-complement units are 5'-phosphorylated and polymerized by ligation in the presence of the non-phosphorylated tail oligonucleotides (A'-H', TABLE 2) as described in Example 4. After four hours at 16°C, the reaction is terminated by heating to 65°C for 15 min, and remaining unligated oligonucleotides are removed by chromatography on Sepharose CL6B (Pharmacia, supra) in the presence of 50% formamide followed by ethanol precipitation.

At least 10 pmoles of each of the polymerized complements to all of the tails are fixed at discrete, known locations to the surface of four nylon membranes as described in Example 4. A mixture of all of the tailed primers is added to four extension reactions, each containing one of the human DNA samples to be tested. The extension reactions are performed using the assay mixture and conditions described in the assay reaction of Example 1 with Sequenase version 2.0 (polymerase*), except that 5 ug of human DNA is used as the template, the primers are not end-labeled, about 1 pmole of each primer is added, 40 uCi of [alpha-^{32}P]dCTP at 3000Ci/mmol without any unlabeled dCTP is used as the source of label, and the temperature for primer annealing is 60°C.

Following the reactions, the presence or absence of extension products is determined as follows. The samples are passed over a Biogel P60 column to remove excess nucleotides, and incubated for 5 min at room temperature after addition of 1/10 volume of 1 M NaOH. Next, they are hybridized to the nylon membranes each of which already has bound to it, at known, distinct positions, a 10-fold excess over the starting primers of polymerized 14-mer complementary to the tails of each of the eight primers. The spots are labeled A'-H' on each of the four membranes corresponding with the locations of each of the poly-complements to the tails A'-H' of TABLE 2. The hybridization reactions are carried out for 30 min at 60°C in 5 ml of 1M NaCl, 0.1 M Tris-HCl, pH 7.5, 1 mM EDTA, 10ug/ml Heparin, 100 ug/ml single-stranded sonicated salmon DNA, and 0.1% sarcosyl after pretreatment of the membrane under the same conditions in the absence of the sample for 30 min. Membrane #1 is hybridized with the products of the reaction with GM6167 DNA, membrane #2 with the products of the reaction with GM7405 DNA, membrane#3 with the products of the reaction with GM7425 DNA, and membrane #4 with the products of the reaction with GM7407A DNA. The membranes are then washed in 2x SSC at room temperature for 20 min followed by 0.2x SSC plus 0.1% SDS at 65°C for 10

min to remove unhybridized material and exposed to x-ray film in the presence of an intensifying screen at -80°C.

The developed film reveals the following results. Membrane #1 produces several spots on the film, with the darkest being coincident with poly-complements to tails A', C', E', and G', consistent with the fact that the human DNA in that reaction mixture is from the normal cell line GM6167. Membrane #2 produces several spots on the film, with the darkest being coincident with poly-complements to tails A', C', F', and G', consistent with the fact that the human DNA in that reaction mixture is from the homozygous IVS-1,110/IVS-1,110 cell line GM7405. Membrane #3 produces several spots on the film, with the darkest being coincident with poly-complements to tails A', C', F', and H', consistent with the fact that the human DNA in that reaction mixture is from the heterozygous IVS-1,110/Codon39 cell line GM7425. Membrane #4 produces several spots on the film, with the darkest being coincident with poly-complements to tails B', D', E', and G', consistent with the fact that the human DNA in that reaction mixture is from the heterozygous IVS-1,6/IVS1,1 cell line GM7407A.

Example 6

Detection of multiple alleles of multiple loci in a single reaction mix

According to this example, the present invention is used to test a human DNA sample for the presence of five alleles of beta-globin (wild-type, IVS1,1, IVS1,6, IVS1,110, and Codon39, as in Example 5) and for the presence of two alleles at another genetic locus identified by sequence only. All of the tests are performed simultaneously on a given human DNA sample in a single reaction mixture by using the tailing method of this invention.

The following cloned DNA segments, called RFLP probes, are known to identify regions of the human genome which display restriction fragment length polymorphisms (RFLPs): CRI-L114, CRI-L819, CRI-L1210, and CRI-L1238 (Donis-Keller et al., 1987, supra). These regions are known to map to chromosomes 4, 7, 4, and 7, respectively, and reveal polymorphisms in cleavage sites with the enzymes HindIII, HindIII, BglII, and EcoRI, respectively (Donis-Keller et al., 1987, supra).

Tailed primers according to the present invention are constructed to test for the nucleotide changes revealed by these RFLP probes by the following procedure. First, the nucleotide change(s) responsible for the loss and gain of the

respective restriction endonuclease cleavage site is determined. This can be accomplished for every known RFLP probe; however, the four chosen here are representative of the simplest case. This is because they reveal two allele systems in which Southern blots show one large band for one allele and two smaller bands for the second allele, and in which the size of the latter two bands adds up to be the size of the single large band of the first allele. In cases such as these, the cloned RFLP probe is very likely to contain DNA encompassing the polymorphic restriction site. Furthermore, the enzyme sites (HindIII, BglII, or EcoRI) do not contain any CG dinucleotides which might be methylated in human cells. Therefore, restriction maps of the human DNA inserts in the clones should match restriction maps of the human locus determined by Southern blot analysis.

The following methods are applied to the four cloned RFLP probes described above. Similar methods could be applied to other RFLP probes, but if the probes do not fit the above criteria, then additional steps may be necessary in order to locate the polymorphic restriction site. For example, if a cloned probe fails to cover the entire locus revealed on a Southern blot, then it may be necessary to find additional clones that include the

polymorphic site. However, for the four probes of this example, the following methods will suffice.

First, the region of the polymorphic restriction site is located on the cloned probe with respect to sites for one or two other restriction enzymes flanking the polymorphic region by generating a simple restriction map of the cloned DNA. The same restriction map, except of course for the polymorphic site, is shown to apply to genomic human DNA cut with the same enzymes used for mapping the cloned probe. This is accomplished by analyzing DNA from several individuals by Southern blot hybridized with the labeled probe. If the two restriction maps do not agree for a particular probe, then further work on that probe is abandonned. Agreement of the two maps indicates that the probe does cover the polymorphic region.

Given that one or more of these four probes does cover the polymorphic region, then two possibilities exist: the cloned probe either contains or does not contain the polymorphic restriction site. If it contains the polymorphic site, then DNA sequences of the flanking regions are determined starting from this site. Next, PCR primers (25-mers) flanking the polymorphic site by about 100 bp on each side are synthesized and used to sequence the second allele following

amplification of genomic DNA from an individual who lacks the site (Gyllensten & Erlich, 1988, Proc. Nat'l. Acad. Sci. USA 85: 7652-7656). From this information, primers suitable for use in this invention are synthesized (see below). They are two 40-mers, one ending with a match at the 3' end for the allele with the polymorphic site, and the other ending with a match at the 3' end for the allele which lacks the polymorphic site.

If the cloned probe does not contain the polymorphic site, then another strategy must be used to determine the DNA sequence of this cloned DNA lacking the polymorphic site. DNA sequences of regions around two restriction sites which flank the polymorphic region, but are no more than 5 kb apart, are determined by standard methods known in the art. Then, PCR primers (30-mers) which hybridize to regions no more than 5 kb apart, are synthesized. These are used to amplify genomic DNA from an individual who exhibits the polymorphic site. The sequence of the region around the polymorphic site is then determined and a second set of PCR Primers flanking the polymorphic site by about 100 bp is synthesized as described above. Next, the latter primers are used to sequence the region of the cloned probe DNA which surrounds the missing polymorphic site, and the sequence of the allele

missing the site is then determined. From this information, primers suitable for use in this invention are synthesized as described above.

The polymorphic site revealed by one of the probes, CRI-L114, CRI-L819, CRI-L1210, or CRI-L1238, is chosen for development of a test according to the present invention. Next, two 40-mer oligonucleotides are synthesized which have a sequence complementary to the region carrying the polymorphic restriction site and whose 3' ends coincide with the variable nucleotide responsible for the gain/loss of the polymorphic restriciton site. The two oligos differ in that one of them carries a 3' nucleotide complementary to one allele and the other carries a 3' nucleotide complementary to the second allele. Each will contain a mismatch if hybridized to the other allele.

The two oligos are attached to polymeric tails and polymeric tail complements are prepared according to the methods of Examples 4 and 5 by using the tail units, staggered tail-complement units, and tail-primer bridges of TABLE 3.

TABLE 3

Tail units:		Staggered Complements to Tail units:		Tail-Primer Bridge:	
1'	5'CGATCATTACTAGC3'	1"	5'ATGATCGGCTAGTA3'	1#	5'XXXXXXXGCTAGTA3'
2'	5'CCAACTGGTCAACC3'	2"	5'CAGTTGGGGTTGAC3'	2#	5'XXXXXXXGGTTGAC3'

(where XXXXXXX represents the sequence complementary to the first seven seven nucleotides of the 5' end of the two primers)

As for Example 5, the ten poly-tail complements (derived from staggered complements to tail units A", B", C", D", E", F", G", H", 1", and 2" of TABLES 1 and 2) are attached at known, discrete locations on a nylon membrane according to methods recommended by the manufacturer.

All ten of the tailed primers (A, B, C, D, E, F, G, H, 1, and 2) are added to a single extension reaction with human DNA from cell line GM6167. In three additional reactions, all ten of the tailed primers are added to human DNA from cell lines GM7405, GM7425, and GM7407A. The reactions are carried out as described in Example 5.

The extension products are hybridized to the nylon membrane carrying the poly-tail complements as described in Example 5, and the membrane is washed and exposed to x-ray film also as described in

Example 5. The developed x-ray film reveals the genotype of each cell line by the relative intensities of the spots resulting from hybridization of extension products to the poly-tail complements on the membrane. The genotype at both the beta globin locus and at the locus identified by the RFLP probe is determined in a single reaction for each human cell line by using the tailed primers and the method of this invention.

It is intended that all matter contained in the above description or shown in the accompanying drawings shall be interpreted in an illustrative and not limiting sense.

What is claimed is:

1. A method for detecting the presence or absence of a first nucleotide at a specific location on a strand of DNA comprising,

treating the DNA strand with an oligo-nucleotide primer, the primer capable of pairing with a first portion of the DNA adjacent to the first nucleotide, the primer including a second nucleotide at a position opposite the first nucleotide when the primer and DNA strand are paired, said treatment including conditions causing the primer and DNA strand to pair,

applying conditions to the paired primer-DNA strand that allow the construction of an extension product complementary to a second portion of the DNA strand if there is a match between the first and second nucleotides, but substantially impairs the construction if there is a mismatch, and

detecting the presence or absence of any extension product.

2. A method as claimed in claim 1 wherein the primer terminates at a position at or close to the second nucleotide, and further characterized by selecting a polymerization agent that is incapable of synthesizing an extension product from the paired primer-DNA strand if there is a mismatch between the first and second nucleotides, but that is capable of

synthesizing an extension product if there is a match between the first and second nucleotides, and

treating the paired primer-DNA strand with the polymerization agent and with extension nucleoside triphosphates under conditions that allow the polymerization agent to construct an extension product complementary to a second portion of the DNA strand if there is a match between the first and second nucleotides.

3. A method as claimed in claim 1 wherein said DNA strand is single stranded and wherein said primer and DNA strand are paired by causing them to hybridize.

4. A method as claimed in claim 1 wherein said DNA strand is treated with a primer capable of pairing with a portion of said DNA strand immediately adjacent to said first nucleotide, said primer including and terminating at said second nucleotide.

5. A method as claimed in claim 1 wherein said paired primer-DNA strand is treated with labeled extension nucleoside triphosphates.

6. A method as claimed in claim 1 further characterized by pairing said primer with a DNA strand comprising at least a portion of a gene.

7. A method as claimed in claim 2 wherein said primer has a 3' end and said second nucleotide is at or close to said 3' end and further characterized by selecting a polymerization agent that is incapable of synthesizing an extension product from the 3' end of said primer if there is a mismatch between said first and second nucleotides.

8. A method as claimed in claim 2 wherein said primer has a 5' end and said second nucleotide is at or close to said 5' end and further characterized by selecting a polymerization agent that is incapable of synthesizing an extension product from the 5' end of said primer if there is a mismatch between said first and second nucleotides.

9. A method as claimed in claim 2 further characterized by selecting a polymerization agent having no 3'exonuclease activity.

10. A method as claimed in claim 2 further characterized by selecting a polymerization agent having no 5' exonuclease activity.

11. A method as claimed in claim 1 further characterized by treating said DNA strand with a primer having a tail that is non-complementary with said DNA strand.

12. A method as claimed in claim 11 further characterized by detecting said extension product by attaching to a substrate an oligonucleotide at least in part complementary to said tail,

subjecting said substrate to conditions that would allow the tail of said extension product, if the extension product exists, to hybridize to the complementary oligonucleotide on said substrate,

and detecting whether extension product has hybridized to the complementary oligonucleotide on said substrate.

13. A method as claimed in claim 12 further characterized by attaching to said substrate an oligonucleotide having repeating units of complementation to said tail.

14. A method as claimed in claim 11 further characterized by treating said DNA strand with a primer having a tail that has repeating units that are non-complementary with said DNA strand.

15. A method as claimed in claim 12 further comprising detecting the presence or absence of a plurality of nucleotides at different locations on a plurality of DNA strands by,

treating a preparation of the DNA strands with a plurality of different primers, each of said primers having a unique tail,

attaching unique complementary oligonucleotides at distinct locations on said substrate, each of said unique complementary oligonucleotides being complementary to one and only one of said tails,

subjecting said substrate to conditions that would allow extension product, if it exists, to hybridize via its unique tail to the unique complementary oligonucleotide attached to said substrate, and

detecting whether any extension product has hybridized at each of said distinct locations.

16. A method as claimed in claim 15 further characterized by selecting oligonucleotide primers capable of recognizing polymorphism, each of said primers terminating at a position opposite the position on a DNA strand responsible for polymorphism.

17. A method as claimed in claim 1 further characterized by detecting said extension product by attaching single stranded DNA complementary with said extension product to a substrate, subjecting said substrate to conditions allowing said extension product, if it exists, and said complementary DNA to hybridize, and detecting whether extension product has hybridized to the complementary DNA on said substrate.

18. A method as claimed in claim 1 further comprising amplifying said extension product, if it exists, to assist in determining the presence or absence of said extension product.

19. A method for detecting the genotype of an individual, said genotype being defined at least in part by single nucleotide variation at specific locations on DNA comprising,

treating the DNA with a plurality of unique oligonucleotide primers, each of the primers capable of pairing with a portion of said DNA adjacent to one of said single nucleotides, each of said primers having two ends, one end being a tail unique to each primer and the other end including a nucleotide at a position opposite to said one of said single nucleotides when said primers and DNA are paired,

and said treatment including conditions causing said primers to pair with said DNA,

selecting a polymerization agent that is incapable of synthesizing an extension product if there is a mismatch at said one of said single nucleotides when said primers and DNA are paired,

treating said paired primers and DNA with said polymerization agent and with extension nucleoside triphosphates and under conditions that allow said polymerization agent to construct an extension product complementary to said DNA if there is a match at any of said terminal positions,

applying said extension product, if it exists, to a substrate spotted at distinct locations with unique complimentary oligonucleotides, each of said complementary oligonucleotides being complementary to one and only one of said tails,

subjecting said substrate to conditions that would allow the tail of any extension product to hybridize to the complementary oligonucleotide on said substrate, and

detecting whether extension product has hybridized at each of said spots.

20. An oligonucleotide primer comprising a sequence of nucleotides complementary with at least a portion of a DNA strand and including a nucleotide

position opposite a test nucleotide position on said DNA strand responsible for polymorphism due to nucleotide variation at said test nucleotide position, said primer terminating within four nucleotides of said nucleotide opposite said test position.

21. An oligonucleotide primer as claimed in claim 20 wherein said primer terminates within three nucleotides of said nucleotide opposite said test position.

22. An oligonucleotide primer as claimed in claim 20 wherein said primer terminates within two nucleotides of said nucleotide opposite said test position.

23. An oligonucleotide primer as claimed in claim 20 wherein said primer terminates within one nucleotide of said nucleotide opposite said test position.

24. An oligonucleotide primer as claimed in claim 20 wherein said primer terminates at said nucleotide opposite said test position.

25. An oligonucleotide primer as claimed in claim 20 further characterized in that said oligonucleotide has two ends, one of said ends being at or close to said nucleotide position opposite said test nucleotide and the other end carrying a tail that is non-complementary with said DNA strand.

26. An oligonucleotide as claimed in claim 25 wherein said tail includes repeating sequences.

27. A set of oligonucleotide primers comprising at least two of the primers of claim 20, said primers being complementary with the same DNA strand and including the same nucleotide position opposite said test nucleotide position, but the primers having different nucleotides at the nucleotide position opposite said test position.

28. A set of oligonucleotide primers comprising at least two of the primers of claim 20, said primers being complementary with the DNA of different DNA strands.

29. A set of oligonucleotide primers comprising at least two of the primers of claim 25, each of said primers having a different tail nucleotide sequence.

30. A kit including the primers of claim 29 and a set of oligonucleotides that are complementary to said tails.

31. A mutant polymerization agent incapable of synthesizing an extension product in the presence of a paired primer-DNA strand having a mismatched base pair at or close to an end of said primer.

32. A polymerization agent as claimed in claim 31 having no 3' exonuclease activity.

33. A polymerization agent as claimed in claim 31 having no 5' exonuclease activity.

34. An article of manufacture useful in DNA hybridization studies comprising,

a substrate,

a plurality of a first oligonucleotide attached at a first location to said substrate, said first oligonucleotide having a first sequence non-complementary with genomic DNA, and

a plurality of a second oligonucleotide attached at a second location different from said first location, said second oligonucleotide having a second sequence different from and non-complementary

with said first oligonucleotide and also non-complementary with genomic DNA.

35. An article of manufacture as claimed in claim 34 wherein said first and second sequences are at least 15 nucleotides long.

36. An article of manufacture as claimed in claim 34 wherein said first oligonucleotide comprises a plurality of repeating first sequences and second oligonucleotide comprises a plurality of repeating second sequences.

37. An article of manufacture useful in DNA hybridization studies comprising,

a substrate, and

a plurality of an oligonucleotide attached to said substrate, said oligonucleotide consisting of a repeating sequence of nucleotides and said plurality of an oligonucleotide cross linked to the substrate.

38. A kit comprising,

a plurality of different oligonucleotide primers, each of said different primers having a unique tail sequence that is non-complementary with genomic DNA and,

a plurality of different, complementary

oligonucleotides, each of said different complementary oligonucleotides capable of pairing with only one of said unique tail sequences.

39. A kit as claimed in claim 38 further characterized in that each of said said complementary oligonucleotides is provided in a separate preparation.

40. A kit as claimed in claim 38 further comprising a substrate, each of said complementary oligonucleotides attached at a different location to said substrate.

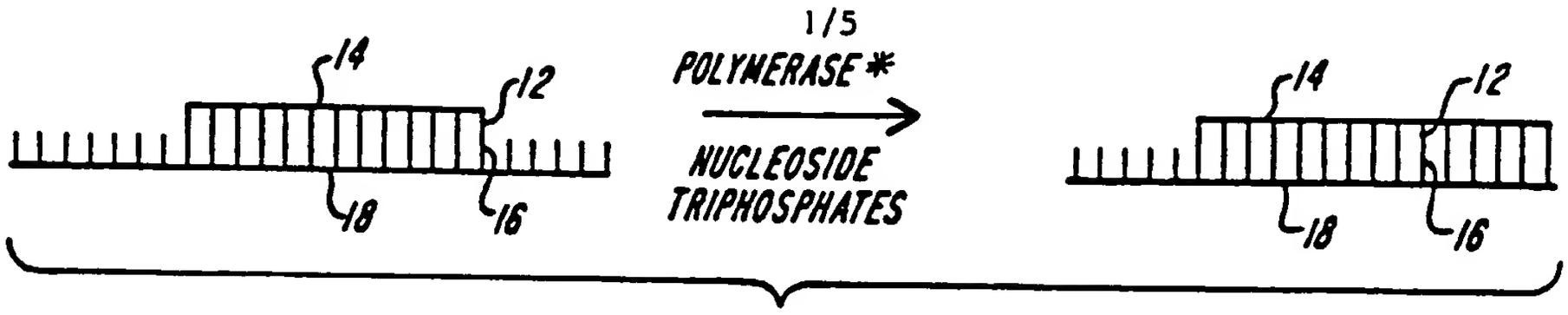


FIG. 1

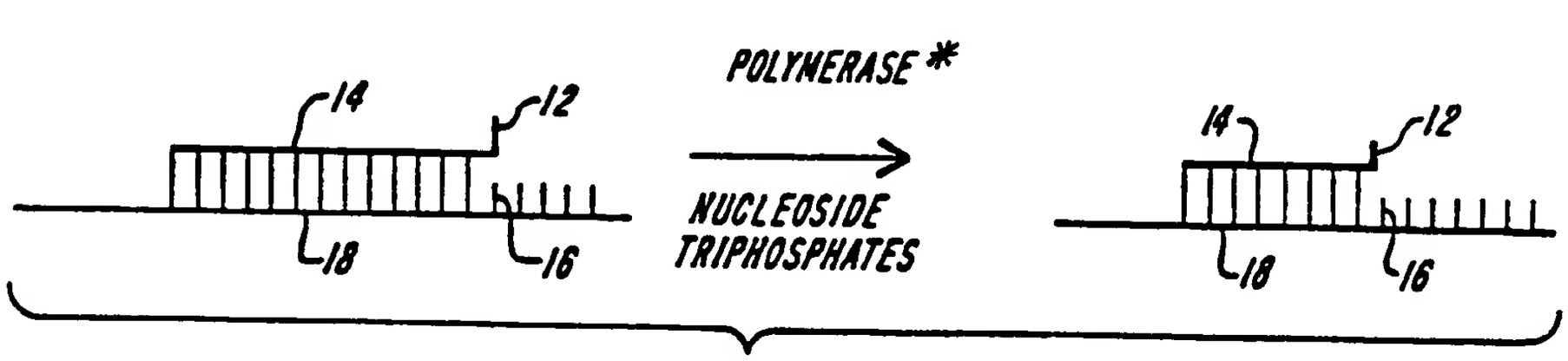


FIG. 2

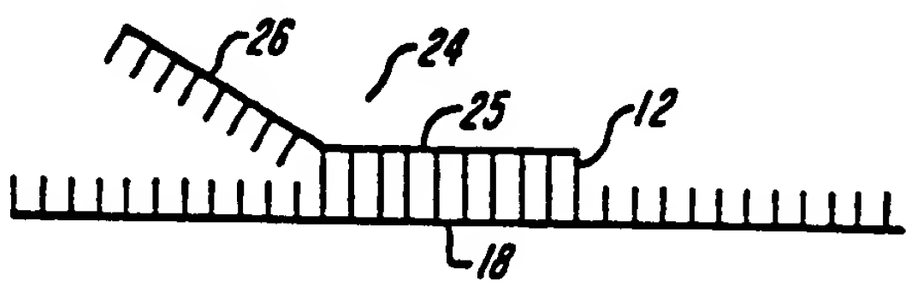


FIG. 3

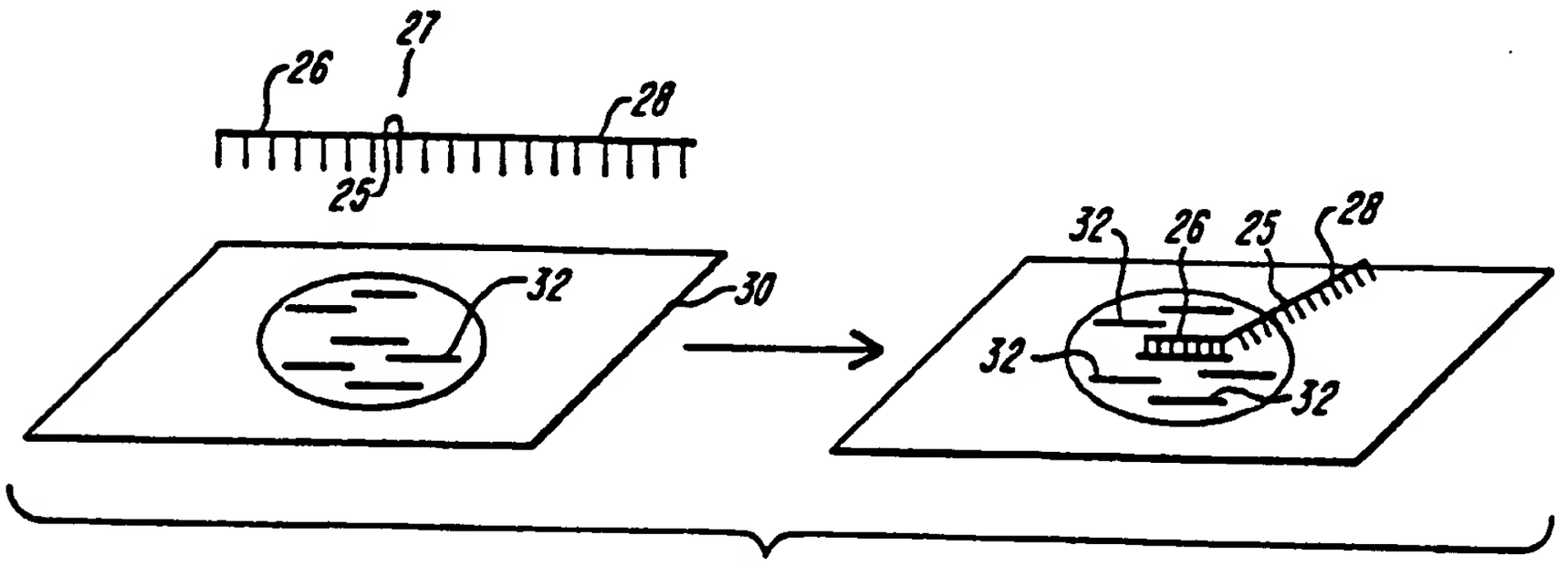


FIG. 4

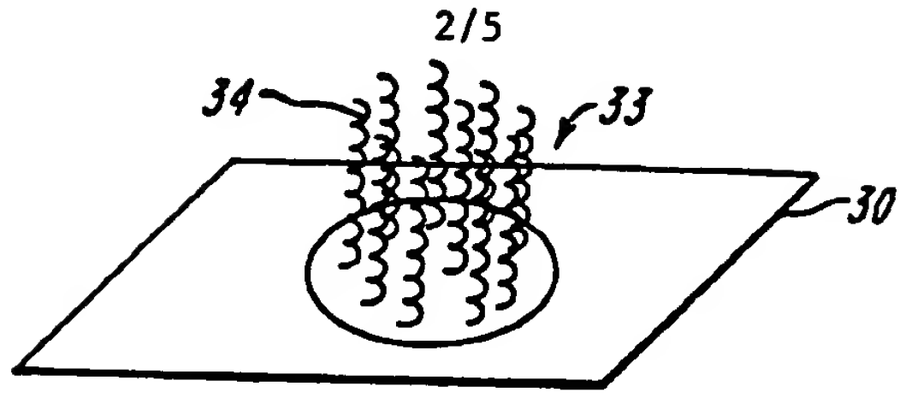


FIG. 5

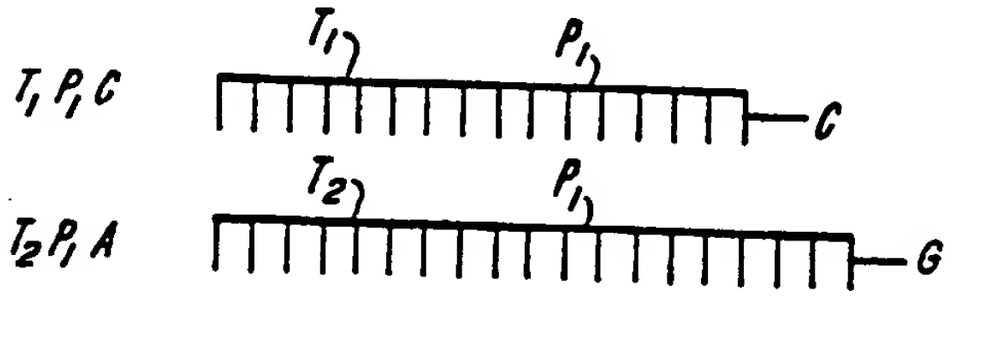


FIG. 6

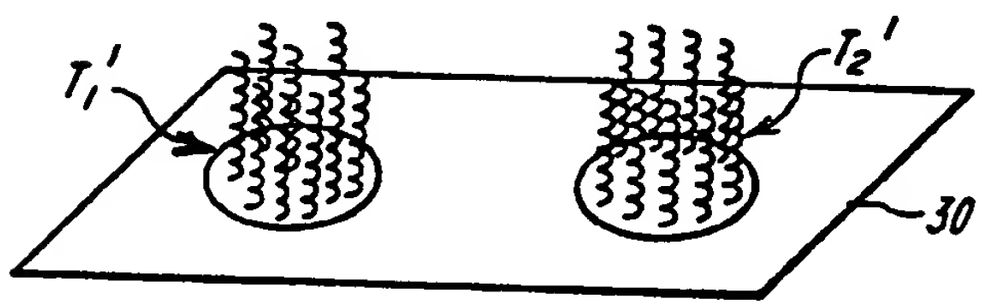
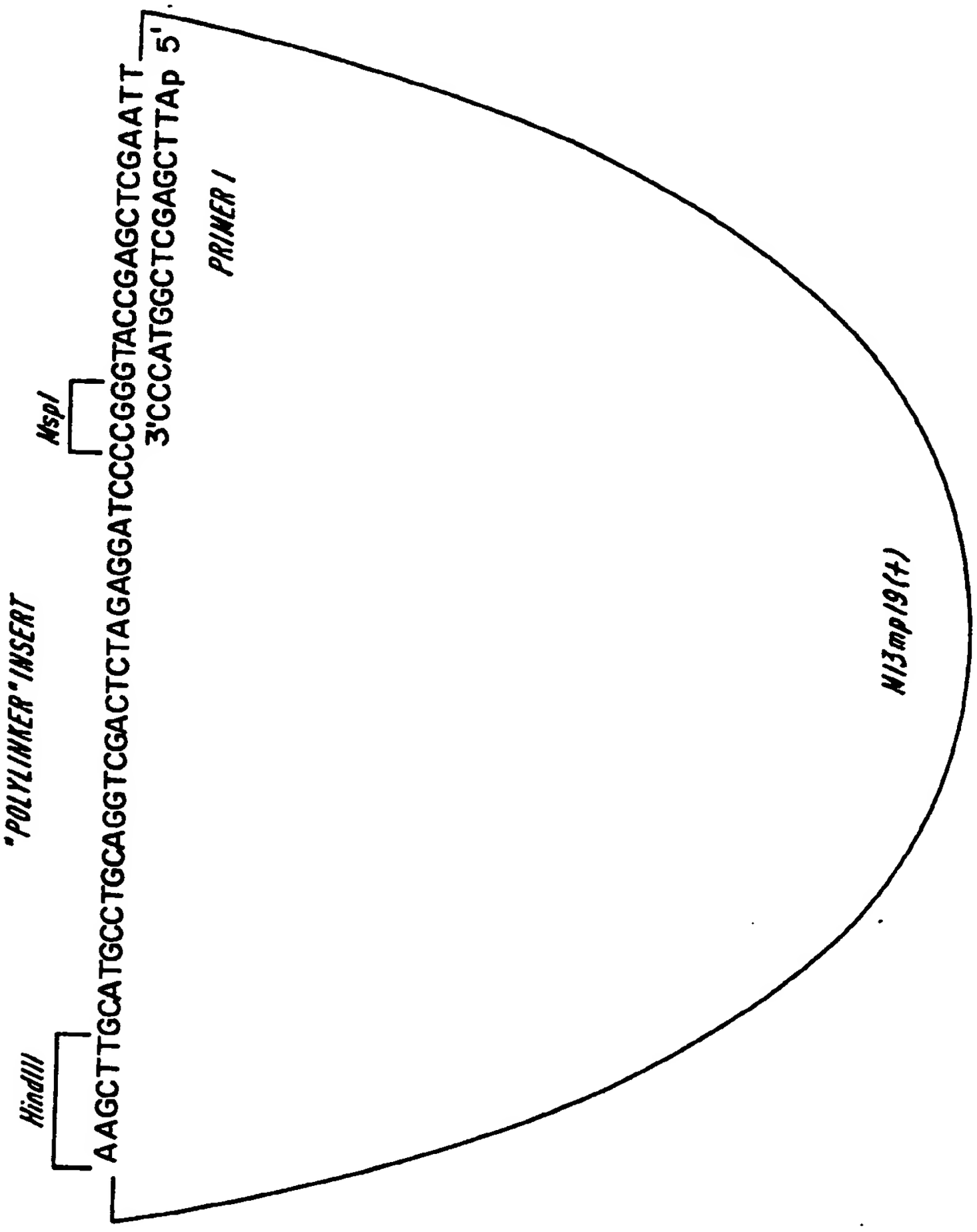


FIG. 7

FIG. 8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Nsp I
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PRIMER I
M13mp19(+)

FIG. 9

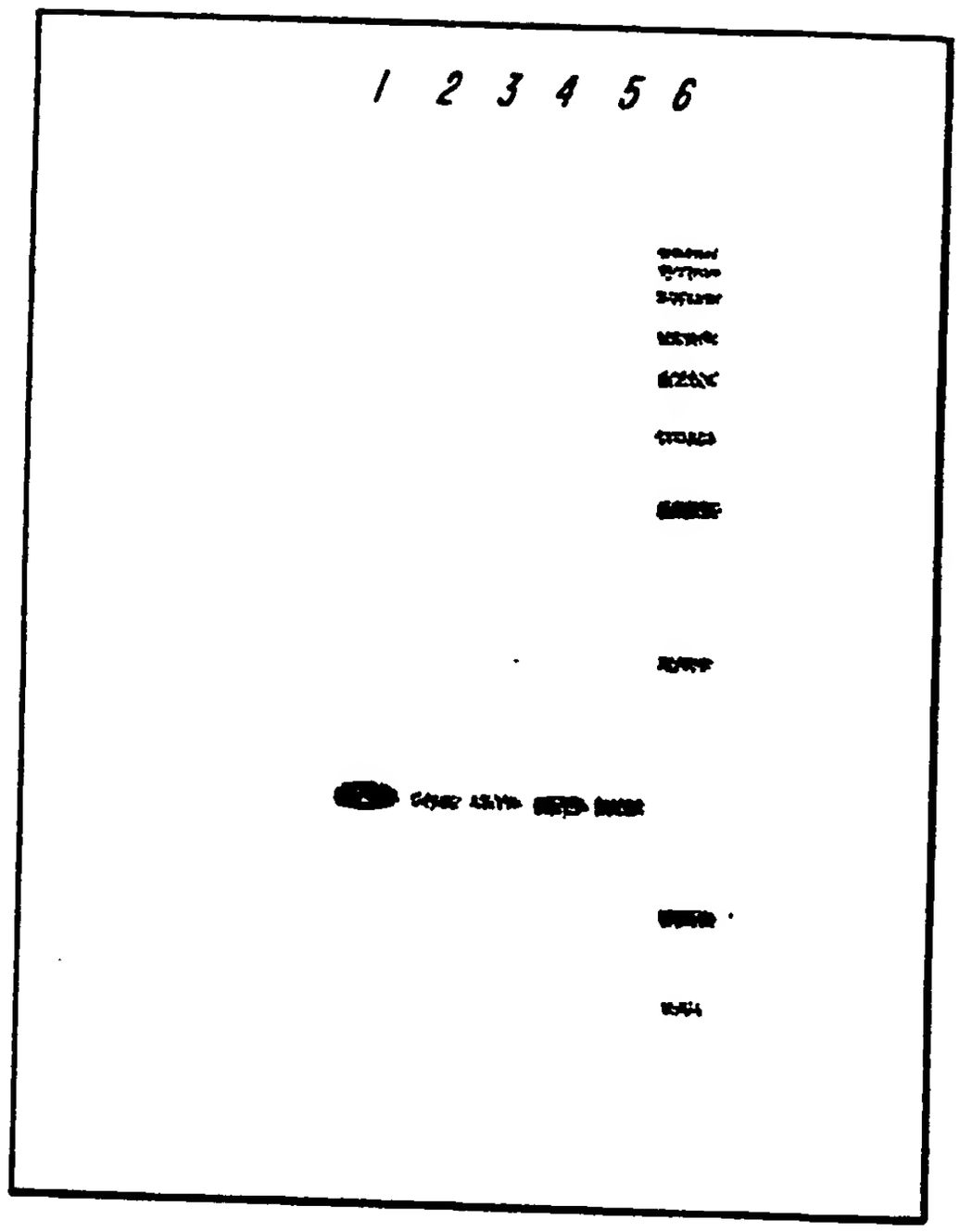
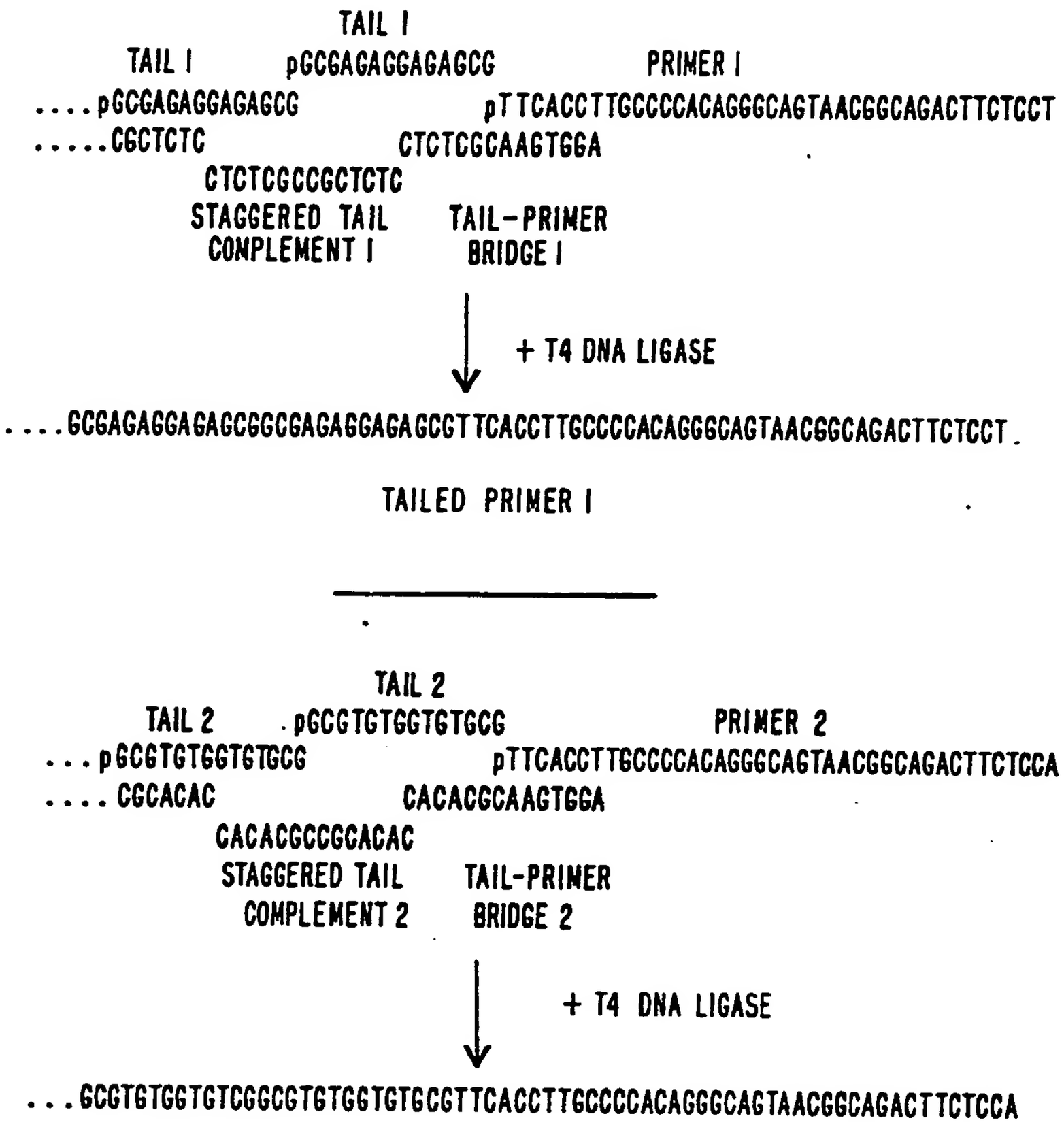


FIG. 10


TAIL 1
TAIL 1
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PRIMER 1
....pGCGAGAGGAGAGCG
pTTCACCTTGCCCCACAGGGCAGTAACGGCAGACTTCTCCT
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CTCTCGCCGCTCTC
STAGGERED TAIL COMPLEMENT 1
TAIL-PRIMER BRIDGE 1
+ T4 DNA LIGASE
....GCGAGAGGAGAGCGGCGAGAGGAGAGCGTTCACCTTGCCCCACAGGGCAGTAACGGCAGACTTCTCCT
TAILED PRIMER 1
TAIL 2
TAIL 2
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PRIMER 2
...pGCGTGTGGTGTGCG
pTTCACCTTGCCCCACAGGGCAGTAACGGCAGACTTCTCCA
....CGCACAC
CACACGCAAGTGGA
CACACGCCGCACAC
STAGGERED TAIL COMPLEMENT 2
TAIL-PRIMER BRIDGE 2
+ T4 DNA LIGASE
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TAILED PRIMER 2

FIG. 11

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US90/01531

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C12Q 1/68
U.S. Cl.: 435/6

II. FIELDS SEARCHED

Minimum Documentation Searched [7]	
Classification System	Classification Symbols
U.S.	435/6,91,172.1,291,805 436/501 536/27 935/17,77,78,88

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
A	Nucleic Acids Research, Volume 13, Number 24 published in 1985, J. Taylor et al., "The Rapid generation of oligonucleotide-directed mutations at high frequency using phosphorothioate-modified DNA", pages 8765-8785, see especially the first paragraph of the "Discussion" section on page 8780.	1-40
Y	Proceedings of the National Academy of Sciences (USA), Volume 85, published in December 1988, M. Innis et al., "DNA sequencing with Thermus aquaticus DNA polymerase and direct sequencing of polymerase chain reaction amplified DNA", pages 9436-9440, see especially page 9440, first column, lines 22-43.	1-10,31-33
X	US,A, 4,656,127 (MUNDY) 07 APRIL 1987, see especially column 2, last 7 lines; column 3; column 6, lines 21-38; and Figure 3.	20-24,27,28
Y		31-37
Y	US,A, 4,677,054 (WHITE ET AL.) 30 JUNE 1987, see especially the abstract.	34-40

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
29 JUNE 1990	02 AUG 1990
International Searching Authority	**Signature of Authorized Officer**
ISA/US	ARDIN MARSCHEL

Form PCT/ISA/210 (second sheet) (Rev. 11-87)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	Biochemical and Biophysical Research Communications, Volume 151, Number 1, published on February 29, 1988, D. Simpson et al., "A Method for Specific Cloning and Sequencing of Human HPRT cDNA for Mutation Analysis", pages 487-492, see especially the abstract.	11-16,19,25, 26,29,30, 38-40